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Abstract: Glioblastoma is the most common malignant brain tumor in adults and characterized by a poor prognosis. Glioma cells expressing O(6)-methylguanine DNA methyltransferase (MGMT) exhibit a higher level of resistance toward alkylating agents, including the standard of care chemotherapeutic agent temozolomide. Here, we demonstrate that long-term glioma cell lines (LTL) as well as glioma-initiating cell lines (GIC) express receptors for the immune modulatory cytokine IFN- and respond to IFN- with induction of STAT-3 phosphorylation. Exposure to IFN- induces a minor loss of viability, but strongly interferes with sphere formation in GIC cultures. Furthermore, IFN- sensitizes LTL and GIC to temozolomide and irradiation. RNA interference confirmed that both IFN- receptors, R1 and R2, are required for IFN- -mediated sensitization, but that sensitization is independent of MGMT or TP53. Most GIC lines are highly temozolomide-resistant, mediated by MGMT expression, but nevertheless susceptible to IFN- sensitization. Gene expression profiling following IFN- treatment revealed strong upregulation of IFN- -associated genes, including a proapoptotic gene cluster, but did not alter stemness-associated expression signatures. Caspase activity and inhibition studies revealed the proapoptotic genes to mediate glioma cell sensitization to exogenous death ligands by IFN- , but not to temozolomide or irradiation, indicating distinct pathways of death sensitization mediated by IFN- . Thus, IFN- is a potential adjunct to glioblastoma treatment that may target the GIC population. IFN- operates independently of MGMT-mediated resistance, classical apoptosis-regulatory networks, and stemness-associated gene clusters. Mol Cancer Ther; 13(4); 948-61. ©2014 AACR.

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Interferon-β induces loss of spherogenicity and overcomes therapy resistance of glioblastoma stem cells

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Abbreviations

CNPase, 2', 3'-cyclic nucleotide 3'-phosphodiesterase; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; FGF, fibroblast growth factor; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GIC, glioma-initiating cells; GO, gene ontology; IFN, interferon; IFNAR, interferon receptor; IL, interleukin; JAK, Janus kinase; LTL, long-term cell line; MFL, Mega-FAS-ligand; MGMT, O^6-methylguanine DNA methyltransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; RMA, robust multi-array analysis; RT-PCR, reverse transcriptase PCR; SFI, specific fluorescence intensity; sh, small hairpin; si, small interfering; STAT, Signal Transducers and Activators of Transcription; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; STS, staurosporine; TMZ, temozolomide; TP53, tumor protein p53; TNFSF10/TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XAF-1, XIAP-associated factor 1; XIAP, X-linked inhibitor of apoptosis

ABSTRACT

Glioblastoma is the most common malignant brain tumor in adults and characterized by a poor prognosis. Glioma cells expressing O^6-methylguanine DNA methyltransferase (*MGMT*) exhibit a higher level of resistance towards alkylating agents, including the standard of care chemotherapeutic agent, temozolomide (TMZ). Here, we demonstrate that long-term glioma cell lines (LTL) as well as glioma-initiating cell lines (GIC) express receptors for the immune modulatory cytokine interferon (IFN)-β and respond to IFN-β with induction of STAT-3 phosphorylation. Exposure to IFN-β induces a minor loss of viability, but strongly interferes with sphere formation in GIC cultures. Further, IFN-β sensitizes LTL and GIC to TMZ and irradiation. RNA interference confirmed that both IFN-β receptors, R1 and R2, are required for IFN-β-mediated sensitization, but that sensitization is independent of MGMT or TP53. Most GIC lines are highly TMZ-resistant mediated by *MGMT* expression, but nevertheless susceptible to IFN-β sensitization. Gene expression profiling following IFN-β treatment revealed strong up-regulation of IFN-β-associated genes including a proapoptotic gene cluster, but did not alter stemness-associated expression signatures. Caspase activity and inhibition studies revealed the proapoptotic genes to mediate glioma cell sensitization to exogenous death ligands by IFN-β, but not to TMZ or irradiation, indicating distinct pathways of death sensitization mediated by IFN-β. Thus, IFN-β is a potential adjunct to glioblastoma treatment that may target the GIC population. IFN-β operates independently of MGMT-mediated resistance, classical apoptosis-regulatory networks and stemness-associated gene clusters.

INTRODUCTION

Glioblastomas are characterized by infiltrative growth and resistance to cell death induction. Despite multimodal therapy, tumor progression occurs inevitably, and survival remains in the range of months (1-3). Early therapy failure is associated with the expression of O^6-methylguanine-DNA-methyltransferase (MGMT), a DNA repair protein that accounts for glioma cell resistance by counteracting the effects of alkylating chemotherapy (4). MGMT has become an important molecular marker and is now implemented in clinical diagnostics as predictive biomarker for benefit from alkylating chemotherapy and clinical outcome (5-7). Patients with a non-methylated *MGMT* promoter are prone to therapy failure with standard alkylating chemotherapy, and to date, effective approaches for this large group of patients, comprising more than half of all glioblastoma patients, are still lacking, including dose intensification of TMZ (8).

Recently, a subfraction of glioma cells exhibiting stem cell-like properties (stem-like glioma cells), referred to as glioma-initiating cells (GIC), has been identified (9, 10). GIC are thought to have the ability of self-renewal, tumor initiation and pluripotency, and have been proposed to account for the ultimately lethal nature of glioblastoma. They may contribute to therapy resistance *in vivo* and therefore promote tumor progression. The value of GIC cultures and their *MGMT* status as a model to study resistance to TMZ in glioblastoma has remained controversial. Among a panel of 20 GIC lines, some were sensitive to TMZ, which was associated with low MGMT protein levels, but not *MGMT* promoter methylation, and the *MGMT* promoter status was thus not strongly predictive of response to TMZ (11). High TMZ sensitivity of GIC cultures lacking MGMT expression has been described *in vitro* (12). Finally, the frequency of *MGMT* promoter-methylated alleles in glioblastomas may range from

10% to 90%, but methylated alleles were enriched in GIC cultures (13). Considering the limited activity of current standards of care, new approaches should take into account this novel target cell population.

IFN-β, a member of the interferon class I family, exerts numerous functions for cellular differentiation, cell growth and immune responses. IFN-β signaling is mediated through binding to a type II cytokine receptor, involving heterodimerization of 2 IFN-α/β receptor subunits, IFNAR-1 and IFNAR-2. Down-stream signalling pathways involve the Janus kinase/signal Transducers and Activators of Transcription (JAK/STAT) pathway (14) and lead to accumulation of MxA protein, a GTPase interfering with the cytoskeletal structure (15, 16). MxA induction is an established marker for responsiveness to IFN-α/β (17, 18).

IFN-β has gained great clinical impact in the treatment of multiple sclerosis. Its safety profile in patients with brain disease has therefore been firmly established. Moreover, IFN-α/β were the first agents to show a significant survival benefit in randomized trials in malignant melanoma (19). The mechanisms mediating antitumor effects of IFN may involve direct cytostatic, anti-angiogenic or immune modulatory activities. IFN-β has been reported to sensitize human glioma cell lines to TMZ in a TP53- and MGMT-dependent manner (20, 21). These reports, however, were based on findings in T98G and U251MG, both being TP53 mutant cell lines (22, 23). Moreover, IFN-β has been attributed anti-stem cell properties in glioma models (24). Finally, clinical trials exploring the addition of IFN-β to radiotherapy plus TMZ have been initiated and interpreted as promising (25, 26). These data prompted us to evaluate the potential role of IFN-β as a component of future, innovative treatment approaches for glioblastoma in more detail. In brief, we demonstrate profound anti-GIC activity of IFN-β as well as IFN-β-mediated sensitization to TMZ and irradiation, but exclude

MGMT and TP53 as primary mediators of these effects. IFN-β exposure induces characteristic IFN-β-associated gene clusters, but does not alter the stemness signature of GIC. A proapoptotic gene signature induced by IFN-β is functionally relevant, but mediates neither anti-GIC properties nor sensitivity to TMZ or radiotherapy.

MATERIALS AND METHODS

Materials and cell lines

The human U87MG and T98G glioma cell lines, K562 leukemia cells and MCF-7 breast adenocarcinoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The other long-term cell lines (LTL) were kindly provided by N. de Tribolet (Lausanne, Switzerland). LNT-229 cells cultured in our laboratory express *TP53* wild-type transcriptional activity (23). The generation of LNT-229 cells depleted of TP or overexpressing *MGMT* has been described (23, 27). *MGMT*-silenced LN-18 cells were generated by transfection with pSUPER puro encoding *MGMT* short hairpin (sh) RNA using Metafectene Pro transfection reagent (Biontex Laboratories GmbH, Martinsried, Germany) (28). The GIC cell lines GS-2, GS-5, GS-7, GS-8 and GS-9 have been characterized elsewhere (29). LTL were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine and penicillin (100 IU/ml) / streptomycin (100 μg/ml). GS sphere cultures were maintained in Neurobasal Medium® supplemented with 2% B-27 supplement, 1% GlutaMAX, 20 ng/ml epidermal growth factor, 20 ng/ml fibroblast growth factor and 32 IE/ml heparin. K-562 cells were maintained in RPMI-1640 supplemented with 1 mmol/L sodium pyruvate (Gibco Life Technologies) and 10% fetal calf serum. TMZ was provided by Schering-Plough (Kenilworth, NJ, USA). A stock solution of TMZ at 200 mM was prepared in dimethylsulfoxide (DMSO). Recombinant IFN-β1b was purchased from AbD Serotec (Dusseldorf, Germany), reconstituted to a concentration of 1,000,000 IU/ml with distilled water. The following antibodies were used: STAT-3 and phospho-STAT-3, Caspase-3 and LC3A/B from Cell Signaling (Boston, MA, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Everest Biotech (Oxfordshire, UK); tumor necrosis factor-related

apoptosis inducing ligand (TRAIL) and XIAP-associated factor 1 (XAF-1) from Santa Cruz (Santa Cruz, CA, USA); caspase 8 from Enzo Life Science (Framingdale, NY, USA); Glial fibrillary acidic protein (GFAP) from Dako (Carpinteria, CA, USA); Nestin from Zytomed Systems GmbH (Berlin, Germany); β-III tubulin from Abcam (Cambridge, UK). Mega-Fas-ligand (MFL) was provided by Topotarget (Copenhagen, Denmark). The MxA antibody was kindly provided by O. Haller, MD and G. Kochs, PhD (Department of Virology, University of Freiburg, Germany). All other reagents, including anti- 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) antibody, O^6-benzylguanine (O^6-BG) and salinomycin, were purchased from Sigma (St. Louis, MO, USA). For irradiation experiments, cells were irradiated in a Co-radiation source (Gebrüder Sulzer, Thermische Energiesysteme, 60-Co, Winterthur, Switzerland) at 1, 3 and 5 Gy at 24 h after IFN-β exposure.

Polymerase chain reaction

Total RNA was prepared using the NucleoSpin System (Macherey-Nagel AG, Önsingen SO, Switzerland) and transcribed using random primers (Bioconcept/NEB, Bioconcept, Allschwil, Switzerland) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For reverse transcriptase PCR, the conditions were: 35 cycles, 94°C/45 sec, specific annealing temperature (54°C for *IFNAR-1*, 52°C for *IFNAR-2*, 51°C for *GAPDH*) /45 sec, 72°C/1 min. For real-time (quantitative) RT-PCR, cDNA amplification was monitored using SYBRGreen chemistry on the 7300 Real time PCR System (Applied Biosystems, Zug, Switzerland). The conditions were: 40 cycles, 95°C/15 sec, 60°C/1 min. Data analysis was done using the $\Delta\Delta C_T$ method for relative quantification. The following specific primers were used: *GAPDH* fwd: 5'-CTCTCTGCTCCTCCTGTTCGAC-3', *GAPDH* rv: 5'-TGAGCGATGTGGCTCGGCT-3'; *IFNAR*-1 fwd: 5'-TAT GCT GCG AAA GTC TTC TTG AG-3'; *IFNAR-1* rv: 5'-TCT

TGG CTA GTT TGG GAA CTG TA-3'; *IFNAR*-2 fwd: 5'-TCT TGA GGC AAG GTC TCG CTA-3'; *IFNAR*-2 rv: 5'-CAG GGA TGC ACG CTT GTA ATC-3'; XAF1 fwd: 5'-AGC AGG TTG GGT GTA CGA TG-3'; XAF1 rv: 5'-TGA GCT GCA TGT CCA GTT TG-3'; TRAIL fwd: 5'-TGC GTG CTG ATC GTG ATC TTC-3' ; TRAIL rv: 5'-GCT CGT TGG TAA AGT ACA CGT A-3'.

Flow cytometry, cell cycle and viability assays

Differentiated glioma cells and sphere cultures were detached respectively dissociated using Accutase (PAA Laboratories, Pasching, Austria) and blocked with 2% FCS in phosphate-buffered saline (PBS). The cells were incubated for 30 min on ice using the following PE-labeled antibodies: anti–IFNAR-1 or anti-IFNAR-2 antibodies (PBL interferon source, Piscataway, NJ, USA) for IFNAR experiments and anti-CD133/2-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) for stem cell marker experiments. Flow cytometry was performed with a CyAn® flow cytometer (Beckman Coulter, Nyon, Switzerland). Signal intensity was calculated as the ratio of the mean fluorescence of the specific antibody and the isotype control antibody (specific fluorescence index). Dead cells were gated out. For some analyses, cells were permeabilized by Fix/Perm Buffer Set (Lucerna Chem AG, Biolegend, Luzern, Switzerland). For analysis of cell death, cells were grown in six-well plates, incubated with IFN-β at 150 IU/ml for 24 h, washed in PBS and allowed to grow for 48 h. Annexin (Anx) V-fluorescein isothiocyanate (1:100) and propidium iodide (PI) (50 µg/ml) were added, and fluorescence in a total of 10,000 events (cells) per condition was recorded in a CyAn flow cytometer. AnxV- or PI-positive cells were counted as dead cells, the remaining cells were designated the surviving cell fraction. In some experiments, loss of viability was also confirmed by trypan blue dye exclusion. Autophagic cell death was assessed by immunoblot and immunostaining. In brief, for

the latter, the cells were exposed to IFN-β as indicated and Cytospin samples were prepared, fixed in 4% PFA, blocked in TBS containing 0.2% Triton X100, 5% goat serum and 5% horse serum, and exposed to the primary LC3A/B antibody (Cell Signaling, Boston, MA, USA) overnight at 4°C and 1 h at room temperature for secondary antibodies.

Clonogenicity assays

For LTL, clonogenicity assays were performed by seeding 100 cells (LNT-229, LN-18) per well in 96-well plates, allowed to adhere overnight, and exposed to IFN-β at 150 IU/ml for 24 h in fresh medium. After removal of IFN-β, the cells were exposed to TMZ at the indicated concentrations for 24 h in serum-free medium, followed by an agent-free observation period for 7-14 days in serum-containing medium. Cell density was assessed by crystal violet staining. For sphere cultures, the cells were seeded at 500 cells per well in neurobasal medium and treated consecutively as indicated above in neurobasal medium. Lower cell numbers resulted in inefficient sphere formation. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Initially, we confirmed that crystal violet assay and MTT assay were good surrogate markers of the number of colonies respectively spheres in these assays, but easier to standardize for large scale concentration response analyses.

Immunoblot analysis

The cells were treated as indicated and lysed in lysis buffer containing 50 mM Tris-HCl, 120 mM NaCl, 5 mM EDTA and 0.5% NP-40. Protein (20 µg/lane) was separated on 10% acrylamide gels. After transfer to nitrocellulose (Biorad, Munich, Germany), the blots were blocked in Tris-buffered saline containing 5% skim milk and

0.05% Tween 20 and incubated overnight at 4°C with primary antibodies and 1 h at room temperature for secondary antibodies. Visualization of protein bands was accomplished using horseradish peroxidase-coupled secondary antibodies (Santa Cruz Healthcare, Santa Cruz, CA, USA) and enhanced chemiluminescence (Pierce / Thermo Fisher, Madison, WI, USA).

Immunocytochemistry and immunofluorescence microscopy

For stemness experiments, the cells were exposed to IFN-β (150 IU/ml) for 48 h and cytospin samples were prepared, fixed in 4% PFA and blocked in either blocking solution (Candor Bioscience GmbH, Wangen, Germany) for GFAP, β-III tubulin or CNPase, or PBS containing 10% swine serum and 0.3% Triton X for nestin, and exposed to the primary antibody overnight at 4°C and 30 min at room temperature for secondary antibodies. Detection was performed with DAB detection systems (Dako) for 5 min at room temperature, and slides were mounted in Eukitt (Sigma-Aldrich). Counterstaining was performed with hemalum. For IFNAR receptor staining, cytospin samples of LTL or GIC were prepared as described above. Staining was performed as suggested by the manufacturer, using IFNAR-1 antibody from antibodies-online.com (Atlanta, GA, USA) and IFNAR-2 antibody from Abcam (Cambridge, UK).

RNA interference-mediated gene silencing

For transient transfections, 2.5×10^5 glioma cells were seeded in a six-well plate and transfected with 100 nM of specific or scrambled control small interfering (si) RNA, using Metafectene Transfection reagent (Biontex). siRNA was purchased from Dharmacon / Thermo Fisher (Chicago, IL, USA) using siGENOME SMARTpool targeting human IFNAR-1 and human IFNAR-2. Samples were collected 48 h post transfection and processed for further treatment.

Reporter assay

Dual luciferase/renilla assays were carried out with co-transfection of 150 ng of the specific reporter construct and 20 ng of the renilla reniformis-CMV (pRL-CMV) control plasmid (Promega, Madison, WI, USA). Luciferase activity was normalized to constitutive renilla activity. The pGL2-Luc *MGMT* construct (30) was a kind gift from Dr. S. Mitra (Sealy Center for Molecular Science and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX, USA). The *TP53*-Luc construct has been described elsewhere (31).

Caspase activity assay

Cells were seeded at a density of 1000 cells/well in a six-well-plate and treated as described above with either IFN-β and TMZ alone or in combination with or without ZVAD-fmk, with either MFL (Mega-FAS-ligand) or staurosporine as positive control. Cells were incubated in lysis buffer (25 mM Tris/HCL, 60 mM NaCl, 2.5 mM EDTA, 0.25% NP40) for 10 min, and the substrate Ac-DEVD-amc was added at a concentration of 20 µM. Fluorescence was assessed at 360 nm wavelength every 15 min until extinction of fluorescence (32).

Microarray-based gene expression profiling

Technical details are provided in the supplementary methods. Array records are deposited under GEO accession number GSE53213.

Data analysis

Data are representative of experiments performed three times with similar results. Where indicated, analysis of significance was performed using the two-tailed

Student's t-test. Synergy of irradiation or TMZ and IFN-β was assessed by the fractional product method (33) where indicated and differences of 10% of observed *versus* predicted (additive) effect were considered synergistic. For the analysis of functional gene interactions, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) Version 9.05 at http://string-db.org (34) was used. Highest confidence settings were applied, integrating combined scores higher than 0.900. Cluster analysis was performed by application of the Markov Cluster algorithm (MCL). Disconnected nodes were hidden from the image.

RESULTS

IFNAR are expressed in human glioma cells

In total, 12 LTL and 5 GIC were assessed for mRNA expression of IFNAR-1 and -2 by RT-PCR. All lines expressed transcripts for both receptor subunits (Fig. 1A). IFNAR-1, in contrast to IFNAR-2, was not detected at the surface on any glioma cell line by flow cytometry (Fig. 1B, left panel), but was detected by immunostaining (Fig. 1B, right panel). Permeabilization of the cells or scraping of cells instead of using accutase did not produce a specific signal for IFNAR-1 on flow cytometry either. Specific fluorescence intensity (SFI) values for IFNAR-2 above 3 were never seen in GIC, but in all LTL (data not shown).

IFN-β signalling in glioma cells involves MxA and STAT-3

To ensure that IFN-β induces classical signalling pathways in glioma cells, we investigated whether STAT-3, the proposed signal transducer in response to IFN-β in gliomas, and the IFN-α/β-regulated protein MxA, a classical marker for cellular responsiveness to IFN-β, were induced. Immunoblot confirmed that STAT-3 phosphorylation levels increased shortly after exposure to IFN-β, with a peak at 30-60 min, with no relevant concentration dependency between 50 and 500 IU/ml, in LTL and GIC (Fig. 1C). Likewise, the exposure to IFN-β led to a concentration-dependent increase of MxA protein in LNT-229 (Fig. 1D, upper row) or LN-18 cells (data not shown), in a time-dependent manner (Fig. 1D, lower row), with first marked elevation of protein levels at a concentration of 150 IU/ml at 24 h.

IFN-β induces cell cycle arrest in glioma cells and causes sphere disruption in GIC

Within the concentration and time ranges of our experiments, IFN-β induced a reduction of G_1 cells in all cell lines, associated with increases in S cells or G_2/M cells or both. Moreover, there was an increase in sub-G_1 cells indicating minor cell death induction (Fig. 2A). A moderate induction of necrotic rather than apoptotic cell death was confirmed by AnxV/PI flow cytometry, defined by an increase of the PI-positive fraction (Fig. 2B). Cellular staining and immunoblot for autophagy were negative in response to IFN-β (Fig. 2C, D).

We also assessed a possible modulation of spherogenicity of GIC by IFN-β. When exposed to 150 IU/ml IFN-β, singularized GIC cultures showed a reduced sphere formation at 1,000 – 10,000 cells per well; lower cell numbers did not result in efficient sphere formation, independent of IFN-β exposure (Fig. 2E). Conversely, exposure of fully formed spheres to 150 IU/ml IFN-β led to sphere disaggregation, with lesser and significantly smaller spheres remaining after 2 weeks of culture, as assessed by sphere count and absorbance (Fig. 2F). The single cells detaching from the sphere were viable during the first days after sphere disaggregation, but underwent cell death after a week, as assessed by trypan blue assay (data not shown).

To assess the possible impact of IFN-β on stem cell differentiation, we analysed GS-2, 5, 7 and 9 cells for changes in the expression of different stem cell markers. GS-2 and GS-9 cells expressed CD133, but expression remained unchanged after exposure to 150 IU/ml IFN-β for 48 h. GS-5 and GS-7 did not express CD133 (Fig. 2G). Moreover, IFN-β-treated GS-2, GS-5 and GS-9 cells showed no changes of GFAP, nestin, β-III-Tubulin or CNPase staining (shown for GS-2) (Fig. 2H).

Requirement for IFNAR-1 and IFNAR-2 for IFN-β-mediated sensitization to irradiation and TMZ

To verify the role of IFNAR-1 and IFNAR-2 for IFN-β-mediated signal transduction and sensitization to TMZ and irradiation, we silenced the expression of both receptors by siRNA. Efficacy of gene silencing was assessed by RT-PCR for IFNAR-1, which could not be assessed by flow cytometry (Fig. 1), and by RT-PCR and flow cytometry for IFNAR-2 (Fig. 3A). In the setting of TMZ exposure (Fig. 3B) or irradiation (Fig. 3C), silencing of IFNAR-1 or -2 or both attenuated the sensitizing effect of IFN-β in LNT-229 cells and LN-18 cells, confirming a role for IFNAR-mediated signal transduction pathways in the IFN-β-induced sensitization process. IFNAR-2 gene silencing alone did not abrogate the effect of IFN-β on TMZ activity to the same extent as IFNAR-1 gene silencing alone.

Response to TMZ, irradiation and IFN in GIC lines

Since GIC are proposed to be the major source of tumor relapse and resistance, we next focused on these models. All investigated GIC, except GS-9, expressed MGMT and were highly resistant to TMZ (Supplementary Note 1, Fig. S1). IFN-β had no major effect on cell density of LTL cells, but a significant concentration-dependent impact of IFN-β alone was observed in GIC cells (Fig. 4A), as expected from the data shown in Fig. 2F. To assess whether IFN-β also partially overcomes TMZ resistance of GIC, we pre-exposed the cells to increasing concentrations of IFN-β (Fig. 4B, left panel), or to 150 IU/ml IFN-β for increasing periods of time (Fig. 4B, right panel), prior to treatment with TMZ at EC_{50} concentrations. All investigated GIC lines showed sensitization to TMZ after pre-exposure to IFN-β. Sensitization was significant in all cell lines when treated at a concentration of 150 IU/ml and for an exposure time of 24

h. Exposure of GS-2 cells to IFN-β for 24 h before administration of TMZ induced a strong growth-inhibiting effect on the sphere cultures (Fig. 4C). IFN-β-treated cells showed a decrease in clonogenic survival exceeding 50% compared to TMZ only-treated cells, and exhibited a progressive loss of the sphere structure (see also Fig. 2D). Similar effects, albeit with less impressive disaggregation, were observed for GS-5, GS-7 and GS-9 spheres, where cooperative effects were most prominent when IFN-β was given with TMZ at approximately EC_{50} concentrations.

In the setting of irradiation experiments, GIC cells were not more resistant than LTL, with GS-2 displaying the strongest radioresistance of the panel, and GS-5 the weakest, possibly because of the *TP53* wild-type status of this cell line (29) (Fig. 4D). Pre-exposure to increasing concentrations of IFN-β followed by low dose irradiation at 1 Gy resulted at least in additive inhibition of clonogenic survival. Again, in the rather radioresistant GS-2, a significant impact of IFN-β alone was observed (Fig. 4E, left panel); preexposure to IFN-β before irradiation led to a reduction of cell density fulfilling criteria of synergy, with an additional reduction of cell density of almost 50% when exposed to 150 IU/ml of IFN-β. GS-5 and GS-7 displayed synergistic effects at 150 IU/ml IFN-β; no synergy was determined in GS-9 (Fig. 4E).

Gene expression analyses

Microarray-based gene expression profiling demonstrated IFN-β-dependent significant differential regulation of 509 genes after 6 h of IFN-β treatment in all three glioma cells lines investigated. Gene expression profiling after 24 h revealed significant differential regulation of 522 genes. The combined analysis, comparing the 6 h and 24 h data, resulted in an overlap of 132 differentially expressed genes upon exposure to IFN-β in all three cell lines. In addition to the statistical analysis, where

IFN-β treatment effects were studied globally across all three cell lines, the impact of IFN-β was assessed individually for every single cell line. Single analysis probe lists of all three cell lines were compared at 6 h and 24 h, respectively (Fig. S2), defining overlapping lists of regulated genes as well as lists of genes exclusively regulated in each individual cell line. Multiple transcripts known to be regulated by IFN-β were found, including *STAT1, interferon regulatory factors* (*IRF)* and *Mx1 (MxA)* (Table S1). Moreover, we noted that a number of genes predicted to promote apoptotic signaling were induced by IFN-β, (Table S1), with an overlap of 6 genes annotated to both gene groups. Submission of the differentially expressed IFN-regulated genes to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) identified a single main cluster of genes with strong interaction profile (Fig. S3A). Submission of the differentially expressed apoptosis-related genes revealed 3 main clusters, one cluster involving tumor necrosis factor-related apoptosis-inducing ligand (TRAIL / TNFSF10)-interacting genes (yellow), one involving XIAP-associated factor 1 (XAF1)-interacting genes (blue), both connected via a third cluster centered around STAT1 (green) (Fig. S3B). Induction of TRAIL and XAF1 was confirmed by RT-PCR and immunoblot analysis (Fig. 5). As we had observed differential reaction of the LTL cell line LNT-229 and the GIC cell lines GS-2 and GS-9 in clonogenic growth assays after exposure to IFN-β alone, we performed a gene ontology (GO) analysis of the genes exclusively regulated (FC > 2) in LNT-229, or the GIC lines (Fig. S2, peripheral regions), and identified several down-regulated genes involved in proliferation and cell growth, as well as up-regulated genes involved in negative regulation of proliferation or cell cycle arrest (Table S2). Notably, more genes were regulated in the GIC lines, with 2 main clusters of negative regulators of proliferation interacting mainly through JUN (blue cluster) and IL-8, IL-6 and JAK2 (red cluster) (Fig. S3C).

Altered expression of apoptosis regulating genes does not mediate enhanced clonogenic cell death induced by TMZ or irradiation

To define a role for altered expression of apoptotic machinery genes in the biological effects of IFN-β, we examined whether IFN-β alone or when combined with different death stimuli promoted caspase activation under the conditions that induced synergistic inhibition of clonogenic survival (Figure 4). As a positive control, we used MFL since we previously observed IFN-α-induced sensitization to CD95L although the mechanism remained unclear then (35). MFL, but neither IFN-β nor TMZ, induced the cleavage of caspases 8 or 3 or DEVD-amc-cleaving caspase activity (Fig. 6A, B, C). Similar results were obtained for irradiation assays (data not shown). Preexposure to IFN-β did not result in caspase processing in cells exposed to TMZ either. Cell death in the investigated settings was confirmed by trypan blue dye exclusion (Fig. 6D). Finally, we performed clonogenic cell death assays similar to those in Figure 4 in the absence or presence of the broad spectrum caspase inhibitor, zVAD-fmk, to explore whether caspase inhibition attenuated or abrogated the effects in clonogenic cell death assays of IFN-β or TMZ alone or in combination (Fig. 6E). These assays confirmed the absence of a role for caspases, either upon single agent exposure or combination. The biological activity of zVAD-fmk under these conditions was confirmed by the inhibition of death receptor-mediated apoptosis as previously reported (36).

DISCUSSION

Even after the implementation of TMZ as the first active chemotherapeutic agent when combined with standard radiotherapy, glioblastomas remain a major challenge in the field of neuro-oncology as they often relapse early and follow an invariably fatal clinical course. The latter is also true for the subgroup of patients with *MGMT* promoter methylation who derive most benefit from TMZ (4, 6). Large efforts have been made to improve prognosis by investigating new agents, either at recurrence, but recently more often early in development already in the paradigm of concomitant and adjuvant TMZ plus radiotherapy (3, 37, 38).

Here we asked whether the immune modulatory cytokine, IFN-β, acts on glioma LTL and GIC lines, and sensitizes for the anti-clonogenic effects of TMZ or irradiation. We detected the expression of IFNAR-1 and -2 mRNA in all glioma cells, making them a potential target for IFN-β (Fig. 1A). Although detection of IFNAR-1 protein turned out to be challenging and was possible by immunofluorescence microscopy only, but not by flow cytometry, the biological effects of IFNAR-1 gene silencing were prominent: siRNA-mediated knockdown of the single IFNAR-1 chain inhibited IFN-β-mediated sensitization (Fig. 3), thereby confirming its biological function. Silencing of the IFNAR-2 chain alone inhibited IFN-β signalling only to a moderate level whereas silencing of IFNAR-1 was additionally required to abort the intracellular signal cascade enough to lead to a highly significant reduction in inhibition of clonogenic survival after IFN-β exposure (Fig. 3B,C). This may be due to the fact that, whilst IFNAR-2 holds only ligand-binding capacities, IFNAR-1, mainly inducing the intracellular signalling cascades, has also been described to have weak ligand binding activity, too, and may interact with other proteins in the absence of IFNAR-2 (39, 40). Alternatively, although both siRNA pools reduced receptor mRNA

expression to a similar extend (Fig. 3A), differential protein stability may account for different biological efficiency of gene silencing in these experiments. Responsiveness of the cells was assessed by monitoring the expression of target proteins of the IFN-β signaling pathways (Fig. 1D).

STAT-3 has been shown to be involved in antiproliferative functions when induced by IFN-α/β in human Daudi cells (41) and, in glioma cells, may act as an IFN-β-induced tumor inhibitor through negative modulation of miR-21 (42). Conversely, there is also accumulating evidence that STAT-3 is a driver of the malignant phenotype of glioblastoma (43). In our study, STAT-3 was uniformly phosphorylated in both LTL and GIC cell lines shortly after exposure to IFN-β (Fig. 1C). Thus, the biological consequences of STAT-3 phosphorylation may be context-dependent and need to be interpreted in the natural course or the therapeutic setting in which they are observed.

Since GIC cells cultured under sphere conditions are considered to be a more resistant subpopulation of glioma cells (44-46), we characterized cell cycle progression, cell death induction and sphere formation capacity of GIC in the presence of IFN-β. Similar to LTL, we observed a cell cycle arrest with a reduced G1 phase and an increase of the sub-G1 population, reflecting a moderate increase in the necrotic fraction on AnxV/PI flow cytometry (Fig. 2A,B). Exposure of singularized cells isolated from sphere cultures to IFN-β led to reduced sphere formation; moreover, fully formed spheres exposed to IFN-β for 24 h disaggregated in the first days (Fig. 2E,F), and the cells underwent delayed cell death after more than a week as assessed by trypan blue dye exclusion (data not shown). This sphere disruption effect with delayed cell death induction appears to be a major effect of IFN-β on GIC survival and to play a more important role than early classical apoptotic pathways that were not shown to be activated in the first 48 h post exposure in AnxV/PI flow

cytometry. Accordingly, Affymetrix array data demonstrated up-regulation of several apoptosis-related genes (Table S1, Fig. S3B), but ZVAD-fmk-controlled clonogenicity assays or DEVD-amc-cleaving-assays did again not confirm an induction of classical caspase-determined apoptotic pathways (Fig. 6C,E).

We demonstrated that IFN-β facilitated the loss of clonogenicity induced by the standard chemotherapeutic agent for glioblastoma, TMZ. Since a sensitizing mechanism mediated through MGMT and TP53 had been described (20), we assessed IFN-β effects on a panel of cells with a heterogeneous pattern of *MGMT* and *TP53* status (Supplementary Note 2, Fig. S4A). In our experimental settings, neither *TP53* nor the *MGMT* status was important for the sensitizing effects of IFN-β. Specifically, we did not observe an induction of TP53 expression in cells with TP53 wild-type transcriptional activity (LNT-229) or a down-regulation of *MGMT* expression in *MGMT*-expressing cells (LN-18) (Supplementary Note 2, Fig. S4C,D), nor did we observe MGMT- or TP53-associated changes in clonogenic survival assays, where sensitization by IFN-β was achieved independent of MGMT or TP53 status (Supplementary Note 2, Fig. S4A,B). Finally, Affymetrix chip analyses did neither detect induction of TP53 nor down-regulation of MGMT in the cell line expressing the respective gene products (TP53: LNT-229, GS-2; MGMT: GS-2; data not shown). The sensitization in the presence of MGMT is particularly attractive in view of the urgent clinical need for novel strategies for the majority of glioblastoma patients with tumors without *MGMT* promoter methylation. Moreover, we confirmed that preexposure to IFN-β sensitized different LTL to the anti-clonogenic effects of single doses of irradiation, too (Fig. S4B), and that these effects were mediated by the known receptors of IFN-β, IFNAR-1 and IFNAR-2 (Fig. 3).

GIC lines, which were confirmed here to be highly resistant to TMZ due to *MGMT* expression, appeared to be a special target to IFN-β which impaired sphere formation when administered alone, in the absence of major cell death induction (Fig. 2).

Accordingly, we observed a growth-inhibiting effect of IFN-β in GIC cultures even when applied as a single agent (Fig. 4A), even in *MGMT*-expressing and highly TMZ-resistant models (Fig. 4B-E). These chemosensitizing effects of IFN-β were concentration- and time-dependent, with significant results assessed at a concentration of 150 IU/ml, and a 24 h application (Fig. 4B, E). Of note, the molecular pattern in GIC cells was also diverse with regard to *TP53* status (29), confirming that TP53 is not a major mediator of IFN-β-induced sensitization in glioma cells.

All glioma models investigated here were sensitized at concentrations of IFN-β reached in the serum in clinical settings following intravenous application, where ranges of over 1000 IU/ml were reported at doses of $18x10^6$ IE i.v. in healthy volunteers (47-49). IFN-β has been applied intravenously before in several clinical studies and was shown to be well tolerated without significant side effects (50), making it an interesting candidate for adjuvant glioblastoma therapy, close to practice. Thus, the present laboratory evidence, together with the wealth of data on the safety and tolerability of IFN-β in patients with multiple sclerosis, justifies further clinical trials of IFN-β in combination with radiotherapy and TMZ in patients with newly diagnosed glioblastoma, specifically those patients suffering from *MGMT*-unmethylated tumors.

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Figure legends

Fig. 1. IFNAR-1 and -2 expression and responsiveness to IFN-β in human LTL and GIC. A,B. The cells were assessed for expression of IFNAR-1 and IFNAR-2 mRNA by PCR with GAPDH as control (A) and of protein by cell surface flow cytometry (B, left panel; representative profiles for K562 as a positive control and LNT-229, LN-18, LN-308, GS-2 and GS-9 GIC cells; black curve: isotype control; grey curve: IFNAR antibody), or immunofluorescence staining (B, right panel; MCF-7 and K562 were used as control). C. Phosphorylation levels of STAT-3 in a concentration- (left) and time-dependent (right) manner, shown representatively for LNT-229 cells (upper row), and for GS-7 and GS-9 GIC in a time-dependent manner (lower row). D. Responsiveness to IFN-β was assessed through detection of MxA protein in a concentration- (upper row) and time-dependent (lower row) manner, shown representatively for LNT-229 cells.

Fig. 2. Assessment of IFN-β effects on GIC cells. A,B. LTL or GIC cells were exposed to IFN-β at 150 IU/ml for 24 h, cultured for an additional 48 h in serum-enriched medium, and flow cytometric cell cycle analysis (A) or AnxV/PI flow cytometry (B) were performed. Cell distributions are shown as bar graphs (striped: sub-G1, black: G1, white: S, grey: G2/M). Cell lines in B match those shown in A above. C. LNT-229 or GS-2 cells were exposed to IFN-β (150 IU/ml), ddH_2O aqua (negative control) or salinomycin (SAL) (4 μM) (positive control) for 24 h. D. LNT-229 and GS-2 cells were treated as in C for protein lysates and assessed for LC3 A/B cleavage. GAPDH was used as control. E. The effect of IFN-β (150 IU/ml, 24 h) on sphere formation over a range of 5 – 10,000 single cells was assessed by sphere count and is shown for GS-2 (black square, IFN-β; white diamond, vehicle). Data are

expressed as mean ± SEM (n=6) (***p<0.001). Photomicrographs corresponding to 1,000, 5,000 and 10,000 cells at 150 IU/ml IFN-β or control are included (original magnification x 40). F. The impact of IFN-β (150 IU/ml, 24 h exposure) on established sphere cultures was assessed after a period of 10 days (upper diagram). Composite quantification of the sphere number and size after exposure to IFN-β 150 IU/ml for 24 h was performed by MTT absorbance measurement as a surrogate marker (lower diagram). Data are expressed as mean ± SEM (n=3) (***p<0.001). GS-2 sphere cells were visualized by bright field microscopy with (right) or without (left) IFN-β exposure for 24 h (original magnification x 40). G, H. GS cells were exposed to ddH_2O or IFN-β at 150 IU/ml for 48 h and assessed for the expression of stem cell markers by flow cytometry (black curve: isotype control; grey curve: specific antibody; SFI values displayed in the diagram) or immunostaining (left, ddH_20 control; right, IFN-β exposure; insert, isotype control; Scale bars = 100 µm).

Fig. 3. Abrogation of the sensitizing effect of IFN-β by IFNAR gene silencing. A. Gene silencing was performed by siRNA and confirmed by quantitative RT-PCR for IFNAR-1 and IFNAR-2 (left), and by flow cytometry for IFNAR-2 (right). B. Control-transfected LNT-229 or LN-18 cells, siIFNAR-1-, siIFNAR-2-, or double-knockdown cells were exposed to IFN-β at 150 IU/ml and to TMZ in the respective EC_{50} concentration range for 24 h each, at 48 h post transfection. Cell density was assessed by crystal violet staining after 2 weeks. Sensitizing effect of IFN-β is represented as delta of IFN-β-exposed versus vehicle-exposed cells. C. Control-transfected, siIFNAR-1-, siIFNAR-2-, or double-knockdown LNT-229 (left) or LN-18 (right) cells were exposed to IFN-β at 150 IU/ml at 48 h post transfection, irradiated with 5 Gy and allowed to grow for 2 weeks after after irradiation. The sensitizing

effect of IFN-β is represented as delta of IFN-β-exposed versus vehicle-exposed cells.

Fig. 4. GIC sensitization to TMZ and irradiation by IFN-β

A. LTL or GIC cells were exposed to IFN-β in a concentration-dependent manner and clonogenic survival was assessed by crystal violet (LTL) or MTT (GIC) assay. B. LTL or GIC cells were exposed to IFN-β in a concentration-dependent (left) or time-dependent (right) manner before a 24 h pulse of TMZ in an EC_{50} concentration range. Clonogenic survival was assessed by crystal violet or MTT assay. C. GS-2, GS-5, GS-7 or GS-9 cells pre-exposed to IFN-β (150 IU/ml, 24 h) were treated with TMZ as shown in Fig. 3A and allowed to grow in neurobasal medium for 2 weeks before the assessment of clonogenicity by MTT assay (control: white bar; IFN-β: black bar). Data are expressed as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001, for the effect of IFN-β + TMZ versus TMZ alone). D. GS-2, GS-5, GS-7 or GS-9 cells were irradiated at 1, 3 or 5 Gy and monitored for clonogenic survival. Cell density was assessed by MTT assay after 2 weeks. E. GS-2, GS-5, GS-7 or GS-9 cells were pre-exposed to increasing concentrations of IFN-β for 24 h and subjected to irradiation with 1 Gy. Cell density was assessed after 2 weeks of clonogenic growth in neurobasal medium by MTT assay. Data are expressed as mean ± SEM normalized to untreated cells (*p<0.05, **p<0.01, ***p<0.001, relative to non-irradiated cells; +, synergistic values, defined as > 10% difference between observed and predicted effect). For A, B and D, data are expressed as mean ± SEM (n=3) (*p<0.05, **p<0.01, ***p<0.001) relative to untreated cells.

Fig. 5. Gene clusters induced by IFN-β. The proapoptotic proteins XAF-1 and

TRAIL were assessed by RT-PCR for RNA and by immunoblot for protein at 6 h and 24 h after exposure to IFN-β in increasing doses (time points as assessed by micro-array profiling), in LNT-229 (left) and GS-2 (right).

Fig. 6. Role of apoptosis-regulatory genes in IFN-β-induced sensitization.

A, B. Immunoblots were performed after exposure of LNT-229 (left panel) or GS-2 (right panel) to IFN-β for 6 h (upper row) or 24 h (lower row) and the levels of caspase 8 and 3 were assessed either for IFN-β exposure alone, TMZ alone, or in combination as described. MFL and STS were used as a positive control (MFL: 1 µg/ml; STS: 1µM). C. DEVD-amc cleaving assays were performed after 6 h of exposure (shown for 2h fluorescence measurement). D. Cell death was assessed by trypan blue exclusion after 6 h of exposure to IFN-β at 150 or 300 IU/ml, IFN-β + TMZ (10 µM for LNT-229, 500 µM for GS-2), or MFL and STS as positive controls, as described in C; ddH_2O was used as negative control (white bars, alive cells; black bars, dead cells; normalized to 100% cells). E. Clonogenic cell death assays were performed for LNT-229 and GS-2 and analyzed by crystal violet staining (for LNT-229) or MTT assay (for GS-2) at d10 after exposure to either IFN-β or TMZ alone, or combinations at indicated concentrations, with or without ZVAD-fmk (15 µM).